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FIBROBLAST GROWTH FACTOR-MEDIATED GROWTH REGULATION AND RECEPTOR EXPRESSION IN EMBRYONAL CARCINOMA AND EMBRYONIC STEM CELLS AND HUMAN GERM CELL TUMOURS

Christine L. Mummery [1,*], Marga van Rooyen [1,2], Madelon Bracke [1], Janny van den Eijnden-van Raaij[1], E. Joop van Zoelen [2], and Kari Alitalo [3]

[1] Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht,The Netherlands

[2] Dept. Cell Biology, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

[3] Cancer Biology Laboratory, Depts. of Pathology and Virology, University of Helsinki, 00290 Helsinki, Finland

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Summary: FGFs have been implicated in the induction of mesoderm in amphibian development and are present in the mouse embryo at stages that would be appropriate for a similar function in mammals. Primitive ectoderm would then be the target tissue. We have now characterized changes in the expression of receptors for FGFs during the differentiation of embryonal carcinoma (EC) and embryonic stem (ES) cells from the mouse. These cells resemble those of the inner cell mass and later primitive ectoderm. On Northern blots of mRNA from undifferentiated cells, transcripts for FGF R1, R2 and R3 are expressed. All are upregulated during differentiation of ES cells and are upregulated or remain constant as EC cells differentiate. FGF R4 is only expressed after differentiation to derivatives resembling parietal endoderm. By contrast in human EC cells, FGF R2 is downregulated during differentiation, FGF R1 and FGF R3 are unchanged and FGF R4 is expressed before and after differentiation. In both human and mouse EC cells three members of the FGF family (a FGF, b FGF and k FGF, also known as FGFs 1,2 and 4) are mitogenic in serum-free medium and one (KGF or FGF 7) appears to have no effect on growth although cellular morphology is altered. Differences between human and mouse cells are primarily in the effects of heparin on the FGF-induced response.

Members of the fibroblast growth factor (FGF) family, of which at least seven are now known, are mitogenic for a wide variety of cell types of mesodermal and neuroectodermal origin. In addition they are involved in the regulation of differentiation and have potent neurotrophic and angiogenic activities (reviewed 9,10). The observation that the proteins encoded by at least four members of this gene family can induce Xenopus animal caps in culture to form mesoderm (11,20,25) has raised the possibility that FGFs play a role in mesoderm formation in vivo. Inhibition of FGF-receptor function in intact Xenopus embryos has recently been shown to result in abnormal gastrulation, supporting the view that the FGF-signaling pathway is important for normal embryonic pattern formation (1). RNA expression patterns of the FGF family members (5,6,18,32) have provided circumstantial evidence that FGFs may have a similar function in the mouse embryo, inducing mesoderm to form from ectoderm during gastrulation. The cellular effects of FGFs are known to be mediated by specific cell surface receptors of the tyrosine kinase gene family. Four members of this family are now known: FGF R1, FGF R2, FGF R3 and FGF R4 (see 9,10). To

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investigate whether primitive ectoderm might be a target for FGFs in the embryo, we have examined embryonal carcinoma (EC) and embryonic stem (ES) cells for FGF receptor expression.

EC and ES cells are the undifferentiated stem cells of teratocarcinomas and blastocyst stage embryos respectively. They resemble cells of the inner cell mass and primitive ectoderm both antigenically and biochemically but behave essentially as tumor cells in culture growing rapidly in the absence of exogenous growth factors. They can be induced to differentiate under appropriate conditions to derivatives of all three germ layers, whereupon they lose their malignant phenotype (12). Our results show that the undifferentiated murine cells express some members of the FGF R family and that they are generally upregulated during differentiation. All four receptors are expressed in undifferentiated human EC cells. At least three FGF family members stimulate the growth of undifferentiated cells in serum free medium and some are also expressed by the cells themselves. FGFs are therefore candidate regulators of autocrine growth of EC and ES cells. The implications of these results are discussed.

MATERIALS AND METHODS

Cell culture and differentiation

ES 5 and D3 embryonic stem cells and P19 and F9 mouse EC cells were cultured as described previously (14,15).Differentiation was induced either by addition of retinoic acid (RA) to cells in monolayer culture (times and concentrations as indicated) or, in some cases, with ES cells, by removal of Buffalo Rat liver (BRL) cell conditioned medium from the culture. DbcAMP (2 mM) was added to F9 EC cells with RA as indicated. The cell phenotypes emerging after these differentiation-inducing regimes have been characterized in terms of marker expression previously (14). The human EC cell lines were cultured in α-MEM as Tera 2 clone 13 previously (16) with the exception of Germa I and II (30) which were cultured in Chang's medium.

Cell proliferation rate

A serum-free assay was used to determine whether FGFs affect the growth rate of undifferentiated EC cells. Near confluent culture of P19 and Tera 2 clone 13 EC cells were incubated in phosphate- buffered saline (PBS) without Ca^{2+} and Mg^{2+} containing EDTA (0.74 mg/ml) for 10 min, resuspended in a 1:1 mixture of DMEM: Ham's F 12 with BSA (0.2%), transferrin (10 μg/ml), $Na_2 SeO_3$ (30 nM) and insulin (5 μg/ml) and plated in 6-well culture dishes (100,000 cells per well) coated with laminin (2.5 μg/well) as described by others (24).The cell number per dish was determined daily for 4 days. The proliferation rates during exponential growth were then determined and the relative effect of the presence of FGFs or FCS compared with the absence of serum.

RNA isolation and Northern blot analysis

Total RNA was isolated and poly A^+ selected from cultures at approximately 90% confluence, as described previously (15). The following human plasmids were used to isolate DNA fragments for use as hybridization probes: FGF R1 or flg (p1:1b), a 2.7 kb fragment cloned in pUC 19 (a gift of L. Claesson-Welsh); FGF R2 or bek (pcD 116), a 1.2 kb fragment cloned in pGEM1 (a gift of C.A. Dionne); FGF R3 (pHE8-1), a 2.4 kb fragment cloned in pGEM 3Z f(+) (19) and FGF R4 (pHE6-2) a 2.5 kb fragment cloned in pGEM 3Z f(+) (19).

RESULTS

Expression of FGF Receptors by Northern blot analysis : undifferentiated cells.

EC and ES cells from the mouse express transcripts for FGF R1, R2 and R3 prior to differentiation, as shown in figures 1 and 2. P19 and F9 EC cells (fig.2) have higher levels of FGF R1 and R3 expression than the ES cell lines ES 5 (fig. 1) and D3 (not shown). In both EC and ES cells FGF R2 expression is weak and FGF R4 (3.0 kb) is not expressed. The position of the 28S band is marked in each figure and is clearly close to the 4.4 kb transcripts of R1 and R2 and the 4.5 kb transcript of R3. Background hybridization to this band is evident in the blots showing FGF R4 expression.

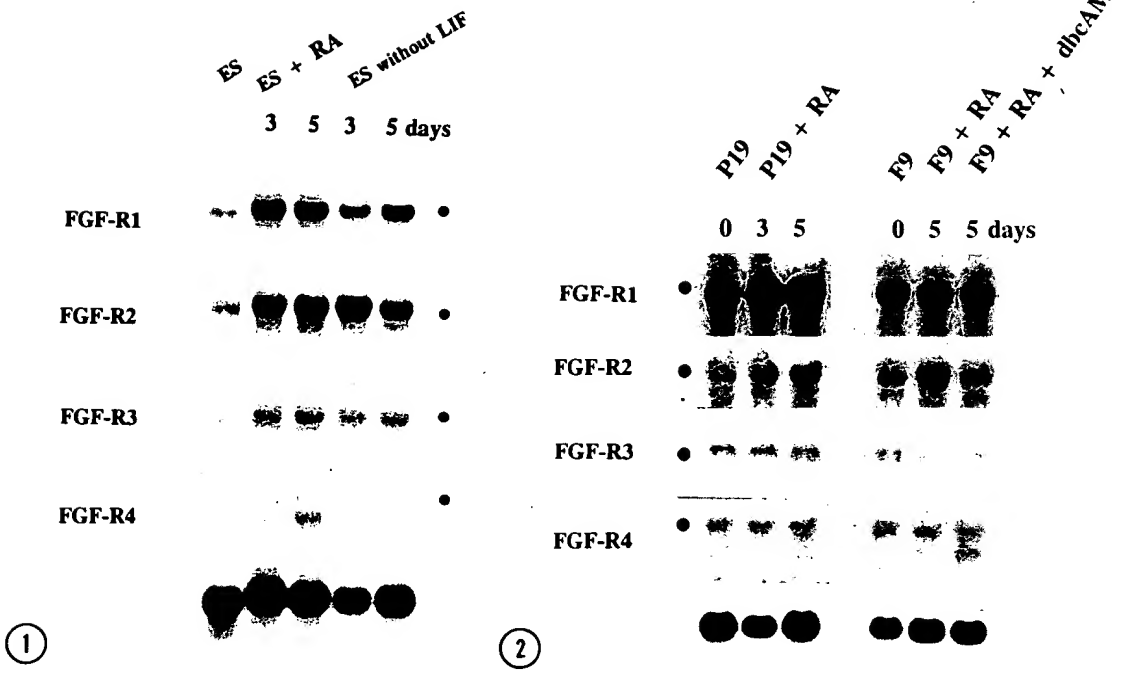


Figure 1.

Expression of FGF receptors in murine ES cells by Northern blot analysis.

Cells are undifferentiated (ES) or induced to differentiate by either addition of RA (10^{-7} M) to BRL-CM for 3 or 5 days or by replacement of BRL-CM by non conditioned medium for 3 or 5 days, as indicated. RNA was isolated, poly A^+ selected and hybridized as described in Materials and Methods. GAPDH is shown as a loading control for each lane.

Figure 2.

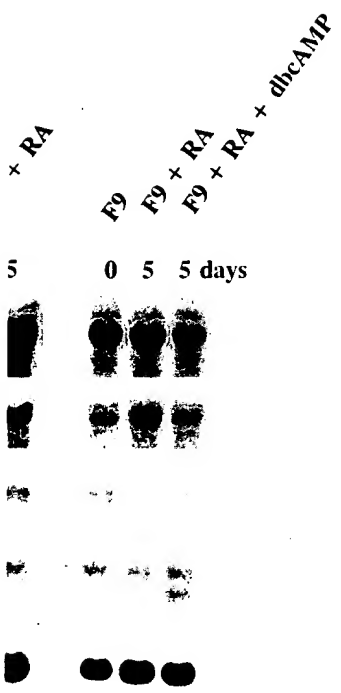
Expression of FGF receptors in murine EC cells.

P19 EC cells were undifferentiated (0) or induced to differentiate with RA (10^{-6} M) for 3 or 5 days as indicated. F9 EC cells were undifferentiated (0) or treated for 5 days with RA ± db cAMP, as indicated. RNA was isolated, poly A^+ selected and hybridized as described in Materials and Methods. GADPH is shown as a loading control for each lane.

By contrast most of the human EC cells lines express all four FGF receptors, with two lines, Germa I and Germa II derived from primary tumors (30), expressing exceptionally high levels (fig 3). The germ cell tumor derived line MA has recently been reclassified as non-EC (J.W. Oosterhuis, personal communication) possibly explaining why this line exceptionally does not express FGFR2 and R3. Additional transcripts for FGF R1 and R2, not present in the mouse cell lines, are observed in the human lines . GAPDH is shown as a loading control for each lane.

: effect of differentiation.

ES cells normally require the presence of differentiation inhibiting activity in the form of leukemia inhibitory factor (LIF) or BRL cell conditioned medium (26,33) to maintain them in an undifferentiated state. Following addition of RA under these conditions, ES cells will differentiate to a population largely consisting of parietal endoderm while upon removal of the inhibitory activity mesenchymal cells will form (14). Independently of whether parietal endoderm or mesenchymal cells are formed, FGF R1, R2 and R3 are upregulated. However induction of an FGF R4 transcript is restricted to differentiation to parietal endoderm (fig. 1). A more conventional model for parietal endoderm formation, F9 EC cells treated with RA and dbcAMP, confirmed this observation (fig.2). FGF R1 and R3 expression are unchanged when F9 and P19 EC are treated with RA.



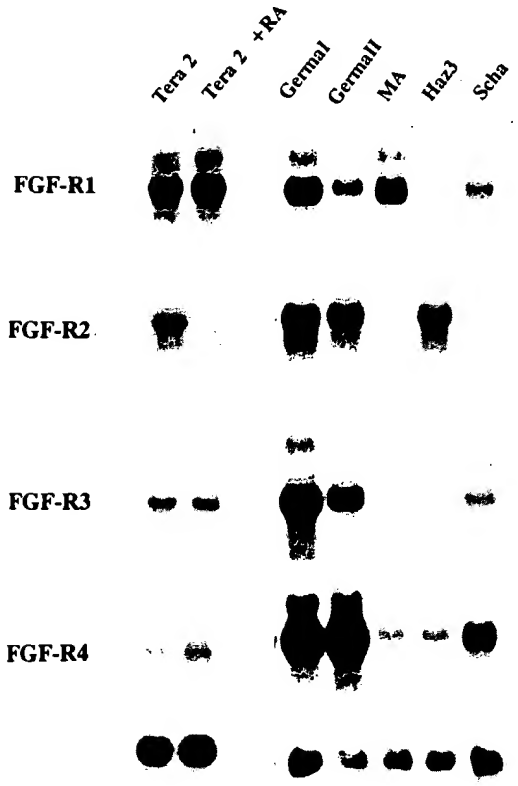


Figure 3.
Expression of FGF receptors in human EC cell lines.
Tera 2 clone 13 cells were undifferentiated (Tera 2) or induced to differentiate with RA (5×10^{-5} M) for 5 days (Tera 2 + RA). The other cell lines were all undifferentiated.

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By contrast RA-induced differentiation of the human EC cell line, Tera 2 clone 13, leads to little change in FGF R1 and R3, R4 expression is slightly increased but there is a striking down regulation of FGF R2.

Effects of FGFs on growth
: P19 EC cells.

It has recently been shown that P19 EC cells plated on a tissue culture substrate in serum-free medium detach and die over a period of several days but that if FGF 2 (basic FGF) is added to the culture medium, they will survive (24). Further, when cells are plated on a laminin- or fibronectin-coated substrate, FGF 2 acts as a mitogen. We have used this assay to begin to assess whether other members of the FGF family are mitogenic for P19 EC cells and whether Tera 2 clone 13 cells are also responsive.

In our hands although P19 EC cells plated on a laminin-coated substrate in serum-free medium attach approximately 50% less efficiently than in the presence of serum, they will proliferate, albeit at a reduced rate (not shown). This reduced proliferation rate is not due to the lower effective plating density since the growth rate of P19 EC is independent of density in this range (not shown). We therefore compared the growth rates determined from the cell number per culture well over a period of 3 days in the presence of various FGFs with that in the basic serum-free medium. The mean of these relative rates over several independent experiments are shown in table 1. In all experiments FGF 1 (acidic FGF) and FGF 2 stimulated the growth of P19 EC and in 3 of 4 experiments FGF 4 (kFGF) also stimulated the growth rate. Heparin enhanced (30%) the effect of FGF1 but not of FGF2

Table 1. Effect of FGFs on the growth of P19 EC cells on laminin in serum-medium

Factor added[1]	Mean increase in growth rate ± standard error of mean: factor above control in serum-free medium (range)	Number of experiments
FCS	2.8 ± 0.4	9
FGF1 (aFGF)[2]	1.3 ± 0.1	3
FGF2 (bFGF)	1.5 ± 0.2	4
FGF4 (kFGF)	1.5 ± 0.3	4
FGF7 (KGF)	0.8 ± 0.2	2
END-2 CM	3.3 ± 0.5	3

[1] FGF1, 2 and 4 added at a concentration of 100 pM
FGF7 (partially purified) added at a 1:100 dilution.

[2] Effect enhanced by heparin (50 μg/ml).

or 4. Preliminary experiments with a partially purified preparation of FGF 7 (KGF) showed no effect on proliferation above the internal control although some cells acquired a more epithelial morphology associated with a loss of alkaline phosphatase (ALP) staining (not shown). Since ALP is a marker for undifferentiated cells (2) differentiation appears to increase under these conditions. All serum-free medium conditioned by a visceral endoderm-like cell line, END-2 (33) however, consistently increased the proliferation rate to a level equivalant that of cells in the presence of serum (table 1).

: Tera 2 clone 13 EC cells.

The human EC cells showed less reduction in attachment efficiency in serum -free media on laminin-coated substrates (not shown) so that, the cell number on day 3 after factor addition could be used to assess for mitogenic activity of FGFs. The results of a typical experiment are shown in figure 4. As for P19 EC cells, FGF 1 and FGF 2 were mitogenic for Tera 2 clone 13 EC cells although, in this case, heparin had either no effect (FGF 1) or was slightly inhibitory (FGF 2). The stimulatory effect of FGF 2 on the growth of undifferentiated Tera 2 clone 13 cells confirmed results of others previously (24). In addition, FGF 4 consistently increased the proliferation rate of the undifferentiated cells; heparin was without effect. The combination of factors in END-2 conditioned medium increased proliferation, as for P19 EC.

DISCUSSION

In the present study, we have investigated the expression of four members of the FGF-R family during the differentiation of cell lines resembling those in the early embryo and have sought to relate this with mitogenic activity of the FGFs themselves. We largely confirmed the results of others (3) that undifferentiated murine and human EC cells express FGF R1 and R2 but also showed that murine ES cells express these receptors as well. These are upregulated or remain constant as the cell lines differentiate but in the human cells, FGF R2 is downregulated as they differentiate. FGF R3 expression is low in the undifferentiated murine cells and FGF R4 is not expressed, in contrast to the human EC cells where expression of both is generally high. The cell lines Germa I and Germa II (30) in particular, which are derived from primary tumors, appear to over-express these receptors. Murine ES cells clearly upregulate FGF R3 mRNA after differentiation although this is less obvious or not the case in P19 and F9 EC. Most striking is the appearance of FGF R4 as F9 EC and ES cells form parietal endoderm. This is in agreement with result from in situ hybridization studies on FGF R4 in murine embryos (27). FGF R4 mRNA was expressed in the definitive endoderm of 8.5 day p.c embryos, as well as in skeletal, but not cardiac, muscle. ES or F9 EC cells differentiating in

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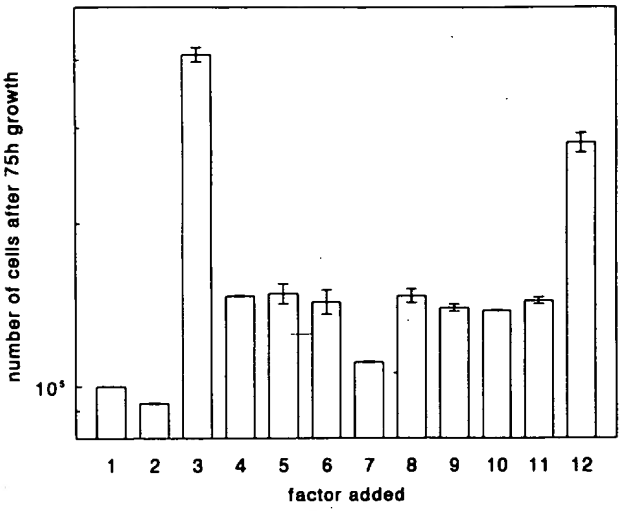

Figure 4.
Effects of FGFs on the growth of Tera 2 clone 13 EC cells on laminin in serum-free medium.
Cells were plated on laminin-coated tissue culture wells at time zero and factors added 6 hrs later, when cells had attached. Cells were counted 75 hrs after plating. Figure shows one of three similar experiments. 1, no. of cells plated at time zero; 2, without serum; 3, with 10% FCS; 4 and 5, FGF1 without and with heparin,respectively; 6 and 7, FGF2 without and with heparin,respectively; 8 and 9, FGF4 without and with heparin,respectively; 10, KGF (partially purified); 11, control for 10; 12, serum-free medium conditioned by END-2 cells. Each point was determined in triplicate. Standard deviation from the mean is indicated for each value.

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Of the four FGF receptors studied here, two (FGF R1 and R2) are known to have multiple splice variants that bind members of the ligand family with different affinity (reviewed 9,10). These splice variants are not distinguishable by Northern blot analalysis. Only single cDNAs have so far been isolated for FGF R3 and FGR R4. FGF 1 (aFGF) binds to all four receptors while FGF2 (bFGF) binds with high affinity to FGF R1 and R3, variable affinity to the variants of R2 and not to R4. FGF 4 (kFGF) binds to R1 and some splice variants of R2 but it is not known whether it binds to the other receptors. Of all the FGF receptors only one, a splice variant of FGF R2, appears to bind one ligand, FGF 7 (KGF), specifically with high affinity; the rest differ primarily in their relative ligand affinities.

To test whether the receptors expressed are functional, we examined undifferentiated EC cells for a mitogenic response to various FGFs in serum free medium, in particular to FGF1. Since this would bind to any receptor present it would act as an internal control for the assay, particularly in the presence of heparin which in many cells enhances the effect of FGF 1 on proliferation, by several possible mechanisms (31 and references therein). In both P19 EC and Tera 2 clone 13 EC cells, FGF1 increased the proliferation rate and in the case of the murine cells, this was enhanced by heparin; we therefore conclude that one or more of the receptors is functional. FGF 2 (bFGF) also enhanced the growth of both cell types, in agreement with previous results on P19 EC (24) but in contrast with results in other murine EC cell lines (21). This discrepancy could possibly be due to differences in the substrate on which the cells in serum free medium were grown; bFGF has been reported only to act as a mitogen for EC cells when they are grown on a laminin substrate (24). Heparin in this case appeared to be without effect in P19 EC cells but to be somewhat inhibitory for the FGF2-induced growth response of Tera 2 clone 13 EC, as reported for other cell types previously (30). FGF 4 (kFGF) stimulated both murine and human EC cells under the conditions tested. This is of interest since EC cells are known to express this factor prior to differentiation whereas after being induced to differentiate they do not (13,22,29). FGF 4 is therefore a candidate for the regulation of autocrine growth in the undifferentiated cells. Recent studies by one of us (Alitalo, in

preparation) have also shown that FGF 1,2 and 4 rapidly and transiently stimulate the expression of cfos, an immediate early transcription factor, in undifferentiated Tera 2 clone 13 cells, confirming the functionality of the FGF-receptors.

In a more limited number of experiments we tested for effects of KGF. A partially purified preparation appeared to have no effect on the proliferation of either P19 or Tera 2 clone 13 EC cells compared with internal controls, although cellular morphology was altered. In addition we tested serum-free medium conditioned by END-2 cells, a visceral endoderm -like derivative of P19 EC that contains activity of an as yet undefined, FGF-like growth factor (34). Stimulation was observed. This showed that endodermal derivatives of P19 EC secrete one or more factors that enhance the proliferation rate of the undifferentiated stem cells, as shown previously for PC 13 EC (8). It is not yet clear whether the FGF-like factor causes this response or other growth factors are involved. In summary these and previous (13,17,21,29) results show that FGF receptors are among the few receptor types expressed by undifferentiated murine EC cells. Apart from FGF 4, we know that P19 EC cells also express FGF 5 (Van den Eijnden-van Raaij et al, unpublished results), but not FGF1, FGF2 or FGF3. FGF4 and FGF5 may therefore be involved in autocrine growth regulation of the murine EC cell lines. Preliminary studies have in fact suggested that, in addition to FGF4, FGF5 (kindly supplied by J. Hébert) is mitogenic for both the mouse and human EC cell lines (not shown). The present results from EC and ES cells also suggest that the ICM and/or primitive ectoderm may express three members of the FGF receptor family and therefore be a potential target for FGFs in the developing embryo.

In contrast to the murine cell lines, human EC cells express in addition to FGF receptors several other classes of growth factor receptor, including those for platelet derived growth factor transforming growth factor β, epidermal growth factor and insulin-like growth factors, although the ligands they express and/or secrete would probably not bind to the particular types of these receptors expressed (reviewed 17). We suggest, therefore, that in human EC cells FGFs are also involved in autocrine growth regulation, with FGF 2 as well as FGF 4 being possible ligands since these are both expressed by the undifferentiated cells (22).

We expect the cells described in this study to provide useful models for studying ligand-receptor interaction and specificity for FGFs, in particular for establishing changes in FGF-mediated signal transduction pathways during differentiation.

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